Skeletal muscle glucose uptake during treadmill exercise in neuronal nitric oxide synthase-μ knockout mice

Hong, YH, Yang, C, Betik, Andrew, Lee-Young, RS and McConell, Glenn (2016) Skeletal muscle glucose uptake during treadmill exercise in neuronal nitric oxide synthase-μ knockout mice. American Journal of Physiology - Endocrinology and Metabolism, 310 (10). E838 - E845. ISSN 0193-1849

SKELETAL MUSCLE GLUCOSE UPTAKE DURING TREADMILL EXERCISE IN NEURONAL NITRIC OXIDE SYNTHASE μ KNOCKOUT MICE

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RUNNING TITLE: Glucose uptake during exercise in nNOSμ KO mice

ABSTRACT

Nitric oxide influences intramuscular signaling that affects skeletal muscle glucose uptake during exercise. The role of the main NO-producing enzyme isoform activated during skeletal muscle contraction, neuronal nitric oxide synthase mu (nNOSμ), in modulating glucose uptake has not been investigated in a physiological exercise model. In this study, conscious and unrestrained chronically catheterized nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ mice either remained at rest or ran on a treadmill at 17 m/min for 30 min. Both groups of mice demonstrated similar exercise capacity during a maximal exercise test to exhaustion (17.7±0.6 vs 15.9±0.9 min for nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ respectively, $P > 0.05$). Resting and exercise blood glucose levels were comparable between genotypes. Very low levels of NOS activity were detected in skeletal muscle from nNOSμ$^{-/-}$ mice and exercise increased NOS activity only in nNOSμ$^{+/+}$ mice (4.4±0.3 to 5.2±0.4 pmol/mg/min, $P < 0.05$). Exercise significantly increased glucose uptake in gastrocnemius muscle (5 to 7-fold) and surprisingly, more so in nNOSμ$^{-/-}$ than nNOSμ$^{+/+}$ mice ($P < 0.05$). This is in parallel with a greater increase in AMPK phosphorylation during exercise in nNOSμ$^{-/-}$ mice. In conclusion, nNOSμ is not essential for skeletal muscle glucose uptake during exercise and the higher skeletal muscle glucose uptake during exercise in nNOSμ$^{-/-}$ mice may be due to compensatory increases in AMPK activation.

Keywords: glucose transport, nitric oxide, AMPK

INTRODUCTION

Skeletal muscle glucose uptake during exercise is an important physiological process for blood glucose and cellular energy homeostasis. It is regulated by intramuscular signaling that modulates membrane permeability to glucose (42). Nitric oxide (NO) is a signaling mediator that can alter membrane permeability to glucose via modulation of GLUT4 translocation (7, 43). The production of NO increases with skeletal muscle contraction/ exercise (4, 27, 45) and a series of studies using NOS inhibitors show that NO mediates skeletal muscle glucose uptake during contraction/ exercise (3, 5, 23, 35, 43, 45). In contrast, some studies found that NO does not play a role in muscle glucose uptake during contraction (7, 12, 21, 46). Methodology differences are believed to contribute to some of the conflicting results (32).

In skeletal muscle, NO from contraction may be derived from several NOS isoforms including endothelial NOS (eNOS) and neuronal NOS (nNOS), which are constitutively expressed in skeletal muscle of rodents (24, 25). Inducible NOS (iNOS) is expressed under inflammatory or disease states (1, 9) and therefore is not likely to be involved in acute contraction-mediated events of animals/ healthy subjects. The most commonly used NOS inhibitors in studies investigating the role of NO in contraction-stimulated glucose uptake, N-G-Monomethyl-L-arginine (L-NMMA) and N-G-Nitro-L-Arginine Methyl Ester (L-NAME), are non-specific competitive inhibitors that inhibit all of the NOS isoforms (54). Therefore, these NOS inhibitors cannot isolate the role of different NOS isoforms in skeletal muscle glucose uptake during contraction/ exercise. As such, genetically modified rodent models are imperative in this regard.

Skeletal muscle glucose uptake during treadmill exercise has previously been determined in $eNOS^{-/-}$ mice which were found to have higher glucose uptake compared with wild type controls (28). This was postulated to be due to the exercise-induced hypoxia in contracting muscle which, in turn, may have stimulated a greater muscle glucose uptake (28) since hypoxia is a potent stimulator of skeletal muscle glucose uptake (6). In addition, NO production during ex vivo contraction was not different between $eNOS^{+/+}$ and $eNOS^{-/-}$ muscles (13) suggesting that eNOS may not be directly involved in NO-mediated intramuscular signaling. Given that nNOSμ is the major NOS isoform activated during contraction (27), it was surprising to find that nNOSμ knockout muscles did not have attenuated muscle glucose uptake during ex vivo contraction (16). Nevertheless, NOS inhibition of isolated nNOSμ knockout ($nNOS\mu^{-/-}$) and wild type ($nNOS\mu^{+/+}$) muscles still attenuated the increase in muscle glucose uptake (16) suggesting that NO was still playing a role in muscle glucose uptake during contraction. It should be considered that ex vivo contraction lacks the complex integrated interactions underlying in vivo exercise conditions such as neural input, blood flow and hormonal changes. Highly relevant to this context is that nNOS has been shown to mediate arterial relaxation in contracting skeletal muscle (27). Thus, in vivo studies are essential to define the role of nNOSμ in muscle glucose uptake during exercise.

In this study, $nNOS\mu^{+/+}$ and $nNOS\mu^{-/-}$ mice were used to investigate the effect of nNOSμ on skeletal muscle glucose uptake in conscious and unrestrained chronically catheterized mice running on a treadmill. This allows examination of the role of nNOSμ in skeletal muscle glucose uptake in a physiological unstressed condition with intact hemodynamic and intramuscular signaling responses. We hypothesized that the increase in muscle glucose uptake during

treadmill running would be attenuated in nNOSμ$^{-/-}$ mice because nNOSμ is the major NOS isoform activated during contraction (27).

MATERIALS AND METHODS

Animals

All procedures were approved by The Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committee, and conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004, 7th Edition). nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ littermates were generated by mating C57Bl/6 nNOSμ$^{+/-}$ mice originally obtained from Jackson Laboratories (Bar Harbor, ME). Genotyping was performed using tail samples obtained at day 21 of age by a commercial vendor (Transnetyx Inc., Cordova, TN). Mice were housed in standard cages and maintained under constant temperature of 21 ± 1^{o}C with 12-hour light/ dark cycle in the AMREP Animal Facility. Animals had access to standard rodent chow and water ad libitum. Both male and female mice were used for experiments at 16 weeks of age.

Exercise stress test

Mice were subjected to an incremental exercise stress test as previously described (29) to determine their maximum exercise capacity. Briefly, two days following a familiarization test (10 m/min for 10 min) mice commenced running at a speed of 10 m/min on a 0% incline treadmill. Running speed was increased by 4 m/min every 3 min until mice were exhausted, which was defined as the point whereby mice continuously remained at the back of the treadmill

for more than five seconds despite tail prodding. Treadmill electrical stimulation was not used in any of the tests.

Surgery and experimental procedures

Surgery procedures were performed as previously described (2) except that only jugular vein cannulation was performed due to an observed intolerance of nNOSμ$^{-/-}$ mice to chronic carotid cannulation. Briefly, mice were anaesthetized with 5% isoflurane in oxygen and maintained with 2% isoflurane in oxygen throughout the cannulation procedure. Carprofen was given subcutaneously for pain relief prior to the skin incision. The right jugular vein was cannulated with a silastic catheter. The free end of the catheter was tunneled under the skin to the back of the neck where it was exteriorized. The catheter was kept patent with saline containing 200 U/ml of heparin and 5 mg/ml of ampicillin, and sealed with stainless steel plugs. Mice were housed individually after surgery and body weight was monitored. Mice were used for experiments at least three days post-surgery when they had fully recovered as indicated by normal activity, healthy appearance and weight regained after surgery.

On the day of the experiment, the exteriorized jugular catheter was connected, via a stainless steel connector, with Micro-Renathane tubing approximately one hour prior to the experiment. Mice were then placed in a single lane treadmill to acclimate to the environment. During the experiment, mice remained sedentary or began a single bout of exercise (t = 0 min). Exercise started at 15 m/min (0% incline) for three min and then increased to 17 m/min throughout the rest of the experiment until t = 30 min (28, 46). Sedentary mice were allowed to move freely on the stationary treadmill for 30 min. In all mice, a bolus of 13 μCi of [1,2-^{3}H]2-

deoxy-glucose ([^{3}H]2-DG) was injected into the jugular vein at t = 5 min for evaluation of tissue-specific glucose uptake. At the end of the experiment, mice were anaesthetized with a jugular vein injection of sodium pentobarbital (3 mg). A tail blood sample was immediately obtained for determination of blood glucose levels. The gastrocnemius and superficial vastus lateralis muscles from each limb and the brain were rapidly excised, frozen with liquid nitrogen-cooled tongs and stored at -80^{o}C. A blood sample was collected via cardiac puncture after exercise and used for plasma insulin and lactate determination.

Muscle glucose uptake determination

The determination of muscle glucose uptake was performed as previously described (8). Muscle sample and brain tissue (~30 mg) were homogenized with 1.5 ml of MilliQ water. Phosphorylated [^{3}H]2-DG ([^{3}H]2-DG-6-P) was extracted from an aliquot of centrifuged homogenates (6000 rpm for 10 min at 4^{o}C) using an anion exchange resin column (AG1-X8, Bio-Rad). Radioactivity of the samples was determined using a β-counter (Tri-Carb 2800TR; Perkin Elmer, Chicago, IL, USA). Glucose uptake for each muscle was expressed as an index of [^{3}H]2-DG-6-P accumulation in the muscle normalized to [^{3}H]2-DG-6-P in the brain of that mouse, as done previously (8, 11). Brain glucose uptake was used as a control for the integrated plasma [^{3}H]2-DG concentration differences over the duration of the experiments (8) as glucose uptake into the brain except the hypothalamus occurs via passive diffusion that follows glucose concentration gradient between the blood and brain tissue (31). In addition, intracellular glucose phosphorylation under normoglycaemic condition and hexokinase II have no impact on brain

glucose uptake (14, 40). Importantly, [^{3}H]2-DG-6-P in the brain was not different between genotypes.

Blood and plasma biochemistry

Plasma insulin concentrations were determined using an enzyme-linked immunosorbent assay (Mercodia, AB, Uppsala, Sweden) as per manufacturer's instructions. Plasma lactate concentrations were analyzed with the enzymatic method of Lowry and Passonneau (30). Blood glucose levels were determined directly from the tail blood using an ACCU-CHEK Advantage monitor (Roche Diagnostics, Indianapolis, Indiana, US).

Immunoblotting

Immunoblotting was performed using ground frozen gastrocnemius muscle homogenized with 200 times volume of solubilizing buffer (125 mM Tris-HCl [pH 6.8], 4% SDS, 10% glycerol, 10 mM EGTA, 0.1 M DTT and 0.01% bromophenol blue) as described previously (15, 38). Five µg of total protein from whole homogenates were separated on SDS-PAGE gels (Bio-Rad Laboratories, Hercules, CA), which was then wet transferred onto polyvinylidine fluoride (PVDF) membranes. Following membrane blocking with 5% skim milk in TBS solution, they were probed with the following primary antibodies overnight: phospho-AMPKα Thr^{172} (1:1000), phospho-TBC1D1 Ser^{660} (1:1000), AMPKα (1:1000), TBC1D1 (1:500), α-tubulin (1:1000) (Cell Signaling Technology, Danvers, MA, USA); nNOS (1:10,000), eNOS (1:10,000), iNOS (1:2000) (BD Biosciences, San Jose, California, USA); GLUT4 (1:8000) (Thermo Scientific, Rockford, IL, USA), and actin (1:40,000) (Sigma Aldrich, St Louis, MO, USA). Chemiluminescent signal

was developed with ECL substrate (SuperSignal West Femto, Pierce, MA, USA) and it was captured with a charge-coupled device (CCD) camera using Quantity One software (Bio-Rad). Pre-stained molecular weight markers were immediately imaged under white light source without changing the membrane position. To quantify both phosphorylated and total protein abundance, phosphorylation-specific primary antibody signal was first determined and then stripped (62.5 mM Tris-HCl pH 6.8, 2% SDS, 0.8% β-mercaptoethanol), re-blocked and re-probed with primary antibody against the total protein. Loading control proteins were always probed on non-stripped membranes and actin was used for all proteins except GLUT4. Actin and GLUT4 have similar molecular weights and it was not possible to probe both of these proteins without undertaking the stripping process, therefore α-tubulin was used as a loading control for GLUT4 abundance.

NOS activity assay

NOS activity was determined as described previously (29) using radiolabeled L-[^{14}C]arginine. NOS activity was expressed as picomoles of L-[^{14}C]citrulline formed per min, per mg of protein. It was determined based on the difference between samples incubated with and without L-NAME.

Statistical analysis

All data are expressed as means ± SEM. Statistical analysis was performed using SPSS statistical package using one factor ANOVA (genotype) or two-factor ANOVA (genotype and exercise). If there was a significant interaction, specific differences between mean values were

identified using Fisher's least significance test. The significance level was set at $P < 0.05$. No sex-specific differences were observed in muscle glucose uptake during exercise (male vs female: $nNOS\mu^{+/+}$: 1.72±0.23 vs 1.50±0.14, $p > 0.05$; $nNOS\mu^{-/-}$: 1.72±0.10 vs 2.10±0.17, $p > 0.05$) and therefore, data from male and female mice were pooled and analyzed together.

RESULTS

Body weight and exercise capacity of $nNOS\mu^{+/+}$ and $nNOS\mu^{-/-}$ mice

At 16 weeks of age, the body weight of $nNOS\mu^{-/-}$ mice was significantly ($P < 0.05$) lower than that of $nNOS\mu^{+/+}$ littermates (Table 1). The ratio of male to female mice was not significantly different in either genotype (Table 1). The maximum running speed achieved during the exercise stress test was similar between genotypes (Table 1). Similarly, the maximum running times were not different between these mice although $nNOS\mu^{-/-}$ mice tended ($P = 0.10$) to run for a shorter time than $nNOS\mu^{+/+}$ littermates (Table 1).

Blood glucose level

At the end of the experiment, blood glucose concentration from the sedentary mice was not significantly different between genotypes (7.9 ± 0.5 mmol/l vs 7.3 ± 0.8 mmol/l for $nNOS\mu^{+/+}$ and $nNOS\mu^{-/-}$ respectively, $P > 0.05$). Exercise had no effect on the blood glucose concentration compared with the sedentary state and remained similar between genotypes (8.7 ± 1.0 mmol/l vs 7.1 ± 0.3 mmol/l for $nNOS\mu^{+/+}$ and $nNOS\mu^{-/-}$ respectively, $P > 0.05$).

Skeletal muscle glucose uptake

Gastrocnemius muscle glucose uptake at rest (sedentary state) was not different between genotypes (Fig. 1A). Exercise significantly increased glucose uptake in gastrocnemius muscle (5 to ~7-fold) and the exercise-induced glucose uptake (fold-increase) was significantly higher in nNOSμ$^{-/-}$ compared with nNOSμ$^{+/+}$ mice ($P < 0.05$) (Fig 1B). A similar muscle glucose uptake pattern was observed in the superficial vastus lateralis (SVL) muscle (Fig 1C & 1D).

Plasma insulin and lactate levels

At the end of the exercise, plasma insulin was not different between genotypes (1.00 ± 0.16 vs 0.89 ± 0.17 μg/l for nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ respectively, $P > 0.05$). Plasma lactate was significantly elevated following exercise compared with the sedentary state (main effect, $P < 0.05$), and the increases following exercise were similar across genotypes (6.0 ± 0.5 vs 5.4 ± 0.7 mmol/l for nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ respectively, $P > 0.05$).

Protein expression and phosphorylation

The expression of actin and α-tubulin proteins was not different between genotypes and they were used as loading controls. Total AMPKα expression in gastrocnemius muscle was not different between genotypes (Fig 2A and 2B). For sedentary muscles, AMPKα Thr^{172} phosphorylation relative to AMPKα abundance was also not different between genotypes. Exercise significantly increased skeletal muscle AMPKα Thr^{172} phosphorylation of both nNOSμ$^{-/-}$ and nNOSμ$^{+/+}$ mice compared with their respective sedentary group (Fig 2C). The increase in

AMPKα Thr^{172} phosphorylation was significantly greater in nNOSμ$^{-/-}$ mice compared with nNOSμ$^{+/+}$ mice (Fig 2C). Expression of TBC1D1 in gastrocnemius muscle was also similar between genotypes (Fig 3A and 3B) and there was no difference in sedentary TBC1D1 Ser^{660} phosphorylation relative to TBC1D1 abundance between genotypes (Fig 3C). Exercise increased TBC1D1 Ser^{660} phosphorylation (Fig 3C; main effect, $P < 0.05$). There was no iNOS detected in either nNOSμ$^{+/+}$ or nNOSμ$^{-/-}$ skeletal muscle. Endothelial NOS (eNOS) (Fig 4A) and GLUT4 (Fig 4B) protein expressions were not different between genotypes.

Expression of nNOSμ, nNOS splice variants and NOS activity

Neuronal NOSμ and nNOSβ (which are splice variants of nNOS) were detected in gastrocnemius muscles of nNOSμ$^{+/+}$ but not nNOSμ$^{-/-}$ mice (results not shown), as we have previously reported in EDL muscles (16). Exercise caused a significant increase in NOS activity in gastrocnemius muscles from nNOSμ$^{+/+}$ mice ($P < 0.05$). Low levels of NOS activity were detected in gastrocnemius muscle from nNOSμ$^{-/-}$ mice (Fig 5) which is in accordance with previous data from EDL muscles (16) and brain tissues of nNOSμ$^{-/-}$ mice (18).

DISCUSSION

In this study we observed that skeletal muscle glucose uptake increased to a significantly greater extent during 30-min of moderate intensity treadmill running in nNOSμ$^{-/-}$ mice than nNOSμ$^{+/+}$ littermates. The higher muscle glucose uptake in nNOSμ$^{-/-}$ mice was observed together with a greater increase in skeletal muscle AMPK phosphorylation during exercise in nNOSμ$^{-/-}$ mice.

Given that NO is involved in GLUT4 translocation and nNOSμ is the main NOS isoform that produces NO during contraction in skeletal muscle (27), it is surprising and interesting to find that glucose uptake during physiological in vivo exercise was enhanced instead of attenuated in mice genetically lacking nNOSμ. Nevertheless, it was recently reported that although NO is involved in mediating skeletal muscle glucose uptake during ex vivo contraction, nNOSμ is not essential in this process (16). Skeletal muscle glucose uptake during ex vivo contraction was normal in mice with or without nNOSμ however glucose uptake was attenuated by NOS inhibition (L-NMMA) in both groups. The reduction in glucose uptake during contraction with L-NMMA was reversed by L-arginine indicating a critical role of NO in mediating glucose uptake in skeletal muscle during ex vivo contraction (16). Under physiological in vivo exercise conditions, various factors beyond the signaling events within the muscle including endocrine, vascular, neural and internal milieu inputs that work in an integrated fashion could affect skeletal muscle glucose uptake.

Neuronal NOSμ$^{-/-}$ mice used in this study were generally comparable with their nNOSμ$^{+/+}$ littermates in a number of phenotypic features that may directly or indirectly influence muscle glucose uptake. The blood glucose level at rest (sedentary) and during exercise was similar in both genotypes implying that the higher glucose uptake in nNOSμ$^{-/-}$ mice was not due to higher blood glucose levels (17). Similarly, plasma insulin levels after exercise were not different between genotypes suggesting that the observed higher glucose uptake in nNOSμ$^{-/-}$ mice was not due to a potential additive effect of insulin on contraction-stimulated glucose uptake (57).

Exercise stimulated a greater muscle AMPK phosphorylation in nNOSμ$^{-/-}$ mice compared with nNOSμ$^{+/+}$ littermates. AMPK is a metabolic fuel sensor that can be activated following metabolic stress/ perturbations in which the degradation of ATP and the consequent

accumulation of ADP and AMP increase the ADP/ATP and AMP/ ATP ratio which leads to an increase in phosphorylation of AMPK (51). The higher AMPK phosphorylation in nNOSμ$^{-/-}$ mice suggests that they may have endured a higher metabolic stress. However, both groups of mice had similar maximum exercise capacity (maximal running speed and time) which suggests that the metabolic stress levels may have been similar. Although not statistically significant, it is possible that the 10% longer running time in the control mice compared with the nNOSμ$^{-/-}$ mice could be important during high intensity exercise. We unfortunately did not measure oxygen uptake or carbohydrate oxidation during this study. Alternatively, AMPK can also be activated under hypoxic conditions (10, 56). nNOS has been shown to be involved in mediating arteriolar relaxation in contracting muscles (27, 50). Therefore, it is plausible that nNOSμ$^{-/-}$ mice might have attenuated blood flow during exercise causing some degree of muscle hypoxia and a higher intramuscular metabolic stress (48) leading to a subsequent increase in phosphorylation of AMPK. It is unfortunate that we were unable to measure blood flow in these mice during exercise due to intolerance of the nNOSμ$^{-/-}$ mice to chronic carotid artery catheterisation. However, eNOS$^{-/-}$ mice with lower exercise-induced increases in blood flow to the contracting muscle and a likely greater hypoxic state in the muscles have no greater increase in AMPK phosphorylation during exercise (28). Indeed, we have shown previously that there is little effect of hypoxia on glucose uptake during exercise in humans (56). Therefore, hypoxia-induced increases in AMPK phosphorylation in nNOSμ$^{-/-}$ mice during exercise appear to be an unlikely stimulus for the greater increase in AMPK phosphorylation during exercise and thus the reasons for this finding remain unclear.

Though the higher muscle glucose uptake in nNOSμ$^{-/-}$ mice could be due to the increased AMPK phosphorylation, we have no direct evidence to prove a causal relationship between these

parameters in nNOSμ$^{-/-}$ mice as we have not investigated glucose uptake during exercise in these mice while preventing the increase of AMPK activation. It may be worthwhile to compare skeletal muscle glucose uptake during ex vivo contraction in nNOSμ$^{-/-}$ muscles that are crossed with an AMPK dominant negative mouse strain.

TBC1D1 has been implicated in the regulation of muscle glucose uptake during contraction/ exercise in which glucose uptake is decreased in muscle overexpressing TBC1D1 mutated on several predicted AMPK phosphorylation sites (53). TBC1D1 Ser^{660} phosphorylation is one of the downstream effectors of AMPK (53) that is stimulated during contraction in mice (53) and exercise in humans (22). The increase in TBC1D1 Ser^{660} phosphorylation with exercise in nNOSμ$^{-/-}$ mice suggests that an AMPK-TBC1D1 mechanism may potentially be involved in stimulating the higher glucose uptake in these mice which, however, remained to be investigated. AMPK can also phosphorylate other downstream mediators such as AS160 to stimulate muscle glucose uptake (26) although there is evidence that AMPK-mediated AS160 phosphorylation does not have a role in muscle glucose uptake during contraction (52).

A caveat to the interpretation of the data using genetically-modified mice needs to be considered. The loss of a protein of interest during development that spans the entire lifespan could possibly induce secondary adaptations including compensatory overexpression of closely related proteins (33). These changes could mask the effects elicited by the loss of the protein of interest. In this study, no compensatory increase in iNOS, eNOS, nNOS splice variants, or GLUT4, all of which could directly or indirectly affect muscle glucose uptake, were detected in nNOSμ$^{-/-}$ mice. Likewise, there was no difference in total AMPK or TBC1D1 expression between genotypes. These data suggest that nNOSμ, similar to ex vivo contraction (16), may not play a role in muscle glucose uptake during in vivo exercise because total loss of nNOSμ did not

attenuate glucose uptake nor elicit a compensatory response in the proteins examined. It should be considered, however, that there may have been compensatory increases in the other potential proteins that may regulate skeletal muscle glucose uptake including Ca^{2+}/ calmodulin-dependent protein kinase (CaMKII) (58), protein kinase C (20), and Rac1/PAK1 (49).

In addition, an exacerbated ROS accumulation during exercise in nNOSμ$^{-/-}$ mice may have contributed to the higher muscle glucose uptake. Muscle contraction/ exercise increases ROS production in the heart and skeletal muscles (41, 47), and ROS increases muscle glucose uptake during ex vivo contraction (36, 47). Following acute exercise, there is significantly higher accumulation of ROS in the myocytes from mice lacking nNOS compared with controls (44). If a similar effect is conferred by nNOS in skeletal muscle during exercise as in the myocytes, it is plausible that muscle glucose uptake in nNOSμ$^{-/-}$ mice could be increased as a result of ROS-induced glucose uptake. Nevertheless, some studies have shown that ROS has no stimulatory effect on muscle glucose uptake during in vivo conditions in rats (34) and humans (37).

The relative roles of nNOSμ could also be affected by the exercise intensity. Given that it has been shown that nNOS is expressed at higher levels in fast-twitch muscles than slow-twitch muscles (24, 36) it would be expected that nNOS would have a greater contribution to glucose uptake during exercise in fast-twitch muscles and/ or at higher exercise intensities. In fact, we have shown that NOS inhibition significantly attenuates the increase in glucose uptake during ex vivo contraction in EDL (mainly fast-twitch) but not in soleus muscles (mainly slow-twitch) (36). However, the fiber type effects on muscle glucose uptake during in vivo exercise are unclear. It is possible that there was no effect of a lack of nNOSμ on glucose uptake during exercise because the intensity of exercise was insufficient to substantially activate nNOSμ. However, the observed increase in NOS activity during exercise suggests that nNOSμ was

indeed activated. Further studies should examine the effects of nNOSμ on glucose uptake during exercise at different intensities.

In this study, we observed very low levels of NOS activity in nNOSμ$^{-/-}$ mice while eNOS abundance was not different between the genotypes. Together with the previous finding that NOS activity is normal or increased in eNOS$^{+/-}$ and eNOS$^{-/-}$ mice, respectively (28), these data indicate that nNOSμ is the predominant NOS isoform responsible for NOS activity in skeletal muscle. This finding is in agreement with a study showing that nNOS is the predominant NOS isoform that activates NO downstream signaling via cGMP during ex vivo contraction (27). Interestingly, eNOS abundance in skeletal muscle was not different between nNOSμ$^{-/-}$ and their wild type littermate control mice in this study, as opposed to our previous study that found a compensatory increase of eNOS expression in nNOSμ$^{-/-}$ muscles (55). However, in that study the control mice were C57Bl/6 mice rather than littermate controls (55). Others have also found no compensation of eNOS expression in myocytes and uterus of mice lacking nNOS when comparing to their wild type littermates (19, 39). This highlights the importance of using littermate controls as a proper experimental control.

In summary, nNOSμ is not essential for skeletal muscle glucose uptake during in vivo exercise. The greater muscle glucose uptake observed in nNOSμ$^{-/-}$ mice than nNOSμ$^{+/+}$ mice during moderate intensity treadmill exercise may be due to the observed greater increase in AMPK activation during exercise.

ACKNOWLEDGEMENTS

The authors would like to thank the members of the Cellular and Molecular Metabolism, Baker IDI Heart & Diabetes Institute, Melbourne, VIC, Australia for their technical assistance in the experiments.

GRANTS

The authors would like to acknowledge funding from the National Health and Medical Research Council (NHMRC) of Australia for support for this study.

AUTHOR CONTRIBUTIONS

Y.H.H., C.Y., A.C.B., R.S.L.Y., and G.K.M. contributed to the conception and design of the research; Y.H.H., C.Y., and R.S.L.Y., performed the experiments; Y.H.H. and A.C.B. analyzed the data; Y.H.H., C.Y., A.C.B., R.S.L.Y., and G.K.M. interpreted the results of the experiments; Y.H.H. and A.C.B. prepared the figures and drafted the manuscript; Y.H.H., C.Y., A.C.B., R.S.L.Y., and G.K.M. edited, revised and approved the final version of the manuscript.

REFERENCES

1. **Adams V, Nehrhoff B, Spate U, Linke A, Schulze PC, Baur A, Gielen S, Hambrecht R, and Schuler G**. Induction of iNOS expression in skeletal muscle by IL-1beta and NFkappaB activation: an in vitro and in vivo study. *Cardiovasc Res* 54: 95-104, 2002.

2. **Ayala JE, Bracy DP, McGuinness OP, and Wasserman DH**. Considerations in the design of hyperinsulinemic-euglycemic clamps in the conscious mouse. *Diabetes* 55: 390-397, 2006.

3. **Balon TW, and Nadler JL**. Evidence that nitric oxide increases glucose transport in skeletal muscle. *J Appl Physiol* 82: 359-363, 1997.

4. **Balon TW, and Nadler JL**. Nitric oxide release is present from incubated skeletal muscle preparations. *J Appl Physiol* 77: 2519-2521, 1994.

5. **Bradley SJ, Kingwell BA, and McConell GK**. Nitric oxide synthase inhibition reduces leg glucose uptake but not blood flow during dynamic exercise in humans. *Diabetes* 48: 1815-1821, 1999.

6. **Cartee GD, Douen AG, Ramlal T, Klip A, and Holloszy JO**. Stimulation of glucose transport in skeletal muscle by hypoxia. *J Appl Physiol* 70: 1593-1600, 1991.

7. **Etgen GJ, Jr., Fryburg DA, and Gibbs EM**. Nitric oxide stimulates skeletal muscle glucose transport through a calcium/contraction- and phosphatidylinositol-3-kinase-independent pathway. *Diabetes* 46: 1915-1919, 1997.

8. **Halseth AE, Bracy DP, and Wasserman DH**. Overexpression of hexokinase II increases insulinand exercise-stimulated muscle glucose uptake in vivo. *Am J Physiol* 276: E70-77, 1999.

9. **Hambrecht R, Adams V, Gielen S, Linke A, Mobius-Winkler S, Yu J, Niebauer J, Jiang H, Fiehn E, and Schuler G**. Exercise intolerance in patients with chronic heart failure and increased expression of inducible nitric oxide synthase in the skeletal muscle. *J Am Coll Cardiol* 33: 174-179, 1999.

10. **Hayashi T, Hirshman MF, Fujii N, Habinowski SA, Witters LA, and Goodyear LJ**. Metabolic stress and altered glucose transport: activation of AMP-activated protein kinase as a unifying coupling mechanism. *Diabetes* 49: 527-531, 2000.

11. **He C, Bassik MC, Moresi V, Sun K, Wei Y, Zou Z, An Z, Loh J, Fisher J, Sun Q, Korsmeyer S, Packer M, May HI, Hill JA, Virgin HW, Gilpin C, Xiao G, Bassel-Duby R, Scherer PE, and Levine B**. Exercise-induced BCL2-regulated autophagy is required for muscle glucose homeostasis. *Nature* 481: 511-515, 2012.

12. **Higaki Y, Hirshman MF, Fujii N, and Goodyear LJ**. Nitric oxide increases glucose uptake through a mechanism that is distinct from the insulin and contraction pathways in rat skeletal muscle. *Diabetes* 50: 241-247, 2001.

13. **Hirschfield W, Moody MR, O'Brien WE, Gregg AR, Bryan RM, Jr., and Reid MB**. Nitric oxide release and contractile properties of skeletal muscles from mice deficient in type III NOS. *Am J Physiol Regul Integr Comp Physiol* 278: R95-R100, 2000.

14. **Hom FG, Goodner CJ, and Berrie MA**. A [3H]2-deoxyglucose method for comparing rates of glucose metabolism and insulin responses among rat tissues in vivo. Validation of the model and the absence of an insulin effect on brain. *Diabetes* 33: 141-152, 1984.

15. **Hong YH, Betik AC, Premilovac D, Dwyer RM, Keske MA, Rattigan S, and McConell GK**. No effect of NOS inhibition on skeletal muscle glucose uptake during in situ

hindlimb contraction in healthy and diabetic Sprague-Dawley rats. *Am J Physiol Regul Integr Comp Physiol* 308: R862-871, 2015.

16. **Hong YH, Frugier T, Zhang X, Murphy RM, Lynch GS, Betik AC, Rattigan S, and McConell GK**. Glucose uptake during contraction in isolated skeletal muscles from neuronal nitric oxide synthase mu knockout mice. *Journal of applied physiology* 118: 1113-1121, 2015.

17. **Howlett K, Angus D, Proietto J, and Hargreaves M**. Effect of increased blood glucose availability on glucose kinetics during exercise. *Journal of applied physiology* 84: 1413-1417, 1998.

18. **Huang PL, Dawson TM, Bredt DS, Snyder SH, and Fishman MC**. Targeted disruption of the neuronal nitric oxide synthase gene. *Cell* 75: 1273-1286, 1993.

19. **Idigo WO, Reilly S, Zhang MH, Zhang YH, Jayaram R, Carnicer R, Crabtree MJ, Balligand JL, and Casadei B**. Regulation of endothelial nitric-oxide synthase (NOS) S-glutathionylation by neuronal NOS: evidence of a functional interaction between myocardial constitutive NOS isoforms. *J Biol Chem* 287: 43665-43673, 2012.

20. **Ihlemann J, Galbo H, and Ploug T**. Calphostin C is an inhibitor of contraction, but not insulin-stimulated glucose transport, in skeletal muscle. *Acta Physiol Scand* 167: 69-75, 1999.

21. **Inyard AC, Clerk LH, Vincent MA, and Barrett EJ**. Contraction stimulates nitric oxide independent microvascular recruitment and increases muscle insulin uptake. *Diabetes* 56: 2194-2200, 2007.

22. **Jessen N, An D, Lihn AS, Nygren J, Hirshman MF, Thorell A, and Goodyear LJ**. Exercise increases TBC1D1 phosphorylation in human skeletal muscle. *Am J Physiol Endocrinol Metab* 301: E164-171, 2011.

23. **Kingwell BA, Formosa M, Muhlmann M, Bradley SJ, and McConell GK**. Nitric oxide synthase inhibition reduces glucose uptake during exercise in individuals with type 2 diabetes more than in control subjects. *Diabetes* 51: 2572-2580, 2002.

24. **Kobzik L, Reid MB, Bredt DS, and Stamler JS**. Nitric oxide in skeletal muscle. *Nature* 372: 546-548, 1994.

25. **Kobzik L, Stringer B, Balligand JL, Reid MB, and Stamler JS**. Endothelial type nitric oxide synthase in skeletal muscle fibers: mitochondrial relationships. *Biochem Biophys Res Commun* 211: 375-381, 1995.

26. **Kramer HF, Witczak CA, Taylor EB, Fujii N, Hirshman MF, and Goodyear LJ**. AS160 regulates insulin- and contraction-stimulated glucose uptake in mouse skeletal muscle. *J Biol Chem* 281: 31478-31485, 2006.

27. **Lau KS, Grange RW, Isotani E, Sarelius IH, Kamm KE, Huang PL, and Stull JT**. nNOS and eNOS modulate cGMP formation and vascular response in contracting fast-twitch skeletal muscle. *Physiol Genomics* 2: 21-27, 2000.

28. **Lee-Young RS, Ayala JE, Hunley CF, James FD, Bracy DP, Kang L, and Wasserman DH**. Endothelial nitric oxide synthase is central to skeletal muscle metabolic regulation and enzymatic signaling during exercise in vivo. *Am J Physiol Regul Integr Comp Physiol* 298: R1399-1408, 2010.

29. **Lee-Young RS, Griffee SR, Lynes SE, Bracy DP, Ayala JE, McGuinness OP, and Wasserman DH**. Skeletal muscle AMP-activated protein kinase is essential for the metabolic response to exercise in vivo. *J Biol Chem* 284: 23925-23934, 2009.

30. **Lowry OH, and Passonneau JV**. *A flexible System of Enzymatic Analysis*. New York: Academic Press, 1972.

31. **Mayer J**. Glucostatic mechanism of regulation of food intake. *N Engl J Med* 249: 13-16, 1953.

32. **McConell GK, and Kingwell BA**. Does nitric oxide regulate skeletal muscle glucose uptake during exercise? *Exerc Sport Sci Rev* 34: 36-41, 2006.

33. **Meeson AP, Radford N, Shelton JM, Mammen PP, DiMaio JM, Hutcheson K, Kong Y, Elterman J, Williams RS, and Garry DJ**. Adaptive mechanisms that preserve cardiac function in mice without myoglobin. *Circ Res* 88: 713-720, 2001.

34. **Merry TL, Dywer RM, Bradley EA, Rattigan S, and McConell GK**. Local hindlimb antioxidant infusion does not affect muscle glucose uptake during in situ contractions in rat. *J Appl Physiol* 108: 1275-1283, 2010.

35. **Merry TL, Lynch GS, and McConell GK**. Downstream mechanisms of nitric oxide-mediated skeletal muscle glucose uptake during contraction. *Am J Physiol Regul Integr Comp Physiol* 299: R1656-1665, 2010.

36. **Merry TL, Steinberg GR, Lynch GS, and McConell GK**. Skeletal muscle glucose uptake during contraction is regulated by nitric oxide and ROS independently of AMPK. *Am J Physiol Endocrinol Metab* 298: E577-585, 2010.

37. **Merry TL, Wadley GD, Stathis CG, Garnham AP, Rattigan S, Hargreaves M, and McConell GK**. N-Acetylcysteine infusion does not affect glucose disposal during prolonged moderate-intensity exercise in humans. *J Physiol* 588: 1623-1634, 2010.

38. **Murphy RM**. Enhanced technique to measure proteins in single segments of human skeletal muscle fibers: fiber-type dependence of AMPK-alpha1 and -beta1. *Journal of applied physiology* 110: 820-825, 2011.

39. **Naghashpour M, and Dahl G**. Relaxation of myometrium by calcitonin gene-related peptide is independent of nitric oxide synthase activity in mouse uterus. *Biology of reproduction* 63: 1421-1427, 2000.

40. **Printz RL, Koch S, Potter LR, O'Doherty RM, Tiesinga JJ, Moritz S, and Granner DK**. Hexokinase II mRNA and gene structure, regulation by insulin, and evolution. *J Biol Chem* 268: 5209-5219, 1993.

41. **Reid MB, Shoji T, Moody MR, and Entman ML**. Reactive oxygen in skeletal muscle. II. Extracellular release of free radicals. *J Appl Physiol* 73: 1805-1809, 1992.

42. **Richter EA, and Hargreaves M**. Exercise, GLUT4, and skeletal muscle glucose uptake. *Physiol Rev* 93: 993-1017, 2013.

43. **Roberts CK, Barnard RJ, Scheck SH, and Balon TW**. Exercise-stimulated glucose transport in skeletal muscle is nitric oxide dependent. *Am J Physiol* 273: E220-225, 1997.

44. **Roof SR, Ho HT, Little SC, Ostler JE, Brundage EA, Periasamy M, Villamena FA, Gyorke S, Biesiadecki BJ, Heymes C, Houser SR, Davis JP, and Ziolo MT**. Obligatory role of neuronal nitric oxide synthase in the heart's antioxidant adaptation with exercise. *Journal of molecular and cellular cardiology* 81: 54-61, 2015.

45. **Ross RM, Wadley GD, Clark MG, Rattigan S, and McConell GK**. Local nitric oxide synthase inhibition reduces skeletal muscle glucose uptake but not capillary blood flow during in situ muscle contraction in rats. *Diabetes* 56: 2885-2892, 2007.

46. **Rottman JN, Bracy D, Malabanan C, Yue Z, Clanton J, and Wasserman DH**. Contrasting effects of exercise and NOS inhibition on tissue-specific fatty acid and glucose uptake in mice. *Am J Physiol Endocrinol Metab* 283: E116-123, 2002.

47. **Sandstrom ME, Zhang SJ, Bruton J, Silva JP, Reid MB, Westerblad H, and Katz A**. Role of reactive oxygen species in contraction-mediated glucose transport in mouse skeletal muscle. *J Physiol* 575: 251-262, 2006.

48. **Suga T, Okita K, Morita N, Yokota T, Hirabayashi K, Horiuchi M, Takada S, Omokawa M, Kinugawa S, and Tsutsui H**. Dose effect on intramuscular metabolic stress during low-intensity resistance exercise with blood flow restriction. *Journal of applied physiology* 108: 1563-1567, 2010.

49. **Sylow L, Jensen TE, Kleinert M, Mouatt JR, Maarbjerg SJ, Jeppesen J, Prats C, Chiu TT, Boguslavsky S, Klip A, Schjerling P, and Richter EA**. Rac1 is a novel regulator of contraction-stimulated glucose uptake in skeletal muscle. *Diabetes* 62: 1139-1151, 2013.

50. **Thomas GD, Shaul PW, Yuhanna IS, Froehner SC, and Adams ME**. Vasomodulation by skeletal muscle-derived nitric oxide requires alpha-syntrophin-mediated sarcolemmal localization of neuronal Nitric oxide synthase. *Circ Res* 92: 554-560, 2003.

51. **Towler MC, and Hardie DG**. AMP-activated protein kinase in metabolic control and insulin signaling. *Circ Res* 100: 328-341, 2007.

52. **Treebak JT, Taylor EB, Witczak CA, An D, Toyoda T, Koh HJ, Xie J, Feener EP, Wojtaszewski JF, Hirshman MF, and Goodyear LJ**. Identification of a novel phosphorylation site on TBC1D4 regulated by AMP-activated protein kinase in skeletal muscle. *Am J Physiol Cell Physiol* 298: C377-385, 2010.

53. **Vichaiwong K, Purohit S, An D, Toyoda T, Jessen N, Hirshman MF, and Goodyear LJ**. Contraction regulates site-specific phosphorylation of TBC1D1 in skeletal muscle. *Biochem J* 431: 311-320, 2010.

54. **Vitecek J, Lojek A, Valacchi G, and Kubala L**. Arginine-based inhibitors of nitric oxide synthase: therapeutic potential and challenges. *Mediators of inflammation* 2012: 318087, 2012.

55. **Wadley GD, Choate J, and McConell GK**. NOS isoform-specific regulation of basal but not exercise-induced mitochondrial biogenesis in mouse skeletal muscle. *J Physiol* 585: 253-262, 2007.

56. **Wadley GD, Lee-Young RS, Canny BJ, Wasuntarawat C, Chen ZP, Hargreaves M, Kemp BE, and McConell GK**. Effect of exercise intensity and hypoxia on skeletal muscle AMPK signaling and substrate metabolism in humans. *Am J Physiol Endocrinol Metab* 290: E694-702, 2006.

57. **Wallberg-Henriksson H, Constable SH, Young DA, and Holloszy JO**. Glucose transport into rat skeletal muscle: interaction between exercise and insulin. *J Appl Physiol* 65: 909-913, 1988.

58. **Wright DC, Hucker KA, Holloszy JO, and Han DH**. Ca2+ and AMPK both mediate stimulation of glucose transport by muscle contractions. *Diabetes* 53: 330-335, 2004.

559 **FIGURE LEGENDS**

560 Figure 1: Gastrocnemius muscle glucose uptake normalized to brain glucose uptake of that same
561 animal (ratio) (A), and relative to sedentary state (fold change) (B), superficial vastus lateralis
562 (SVL) muscle glucose uptake normalized to brain glucose uptake of that same animal (ratio) (C),
563 and relative to sedentary state (fold change) (D). Data are means ± SEM, n = 11 & 3 for
564 sedentary nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ respectively, and 10 & 6 for exercise nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$
565 respectively. * $P < 0.05$ vs sedentary of the same genotype, # $P < 0.05$ vs exercise nNOSμ$^{+/+}$.

566

567 Figure 2: Representative blots for AMPK, AMPKα Thr^{172} phosphorylation and actin (A),
568 gastrocnemius muscle AMPKα abundance in sedentary muscles (B), and gastrocnemius muscle
569 AMPKα Thr^{172} phosphorylation relative to AMPKα abundance (C). Data are means ± SEM, n =
570 9 & 4 for sedentary nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ respectively, and 9 & 5 for exercise nNOSμ$^{+/+}$ and
571 nNOSμ$^{-/-}$ respectively.* $P < 0.05$ vs sedentary of the same genotype; # $P < 0.05$ vs exercise
572 nNOSμ$^{+/+}$.

573

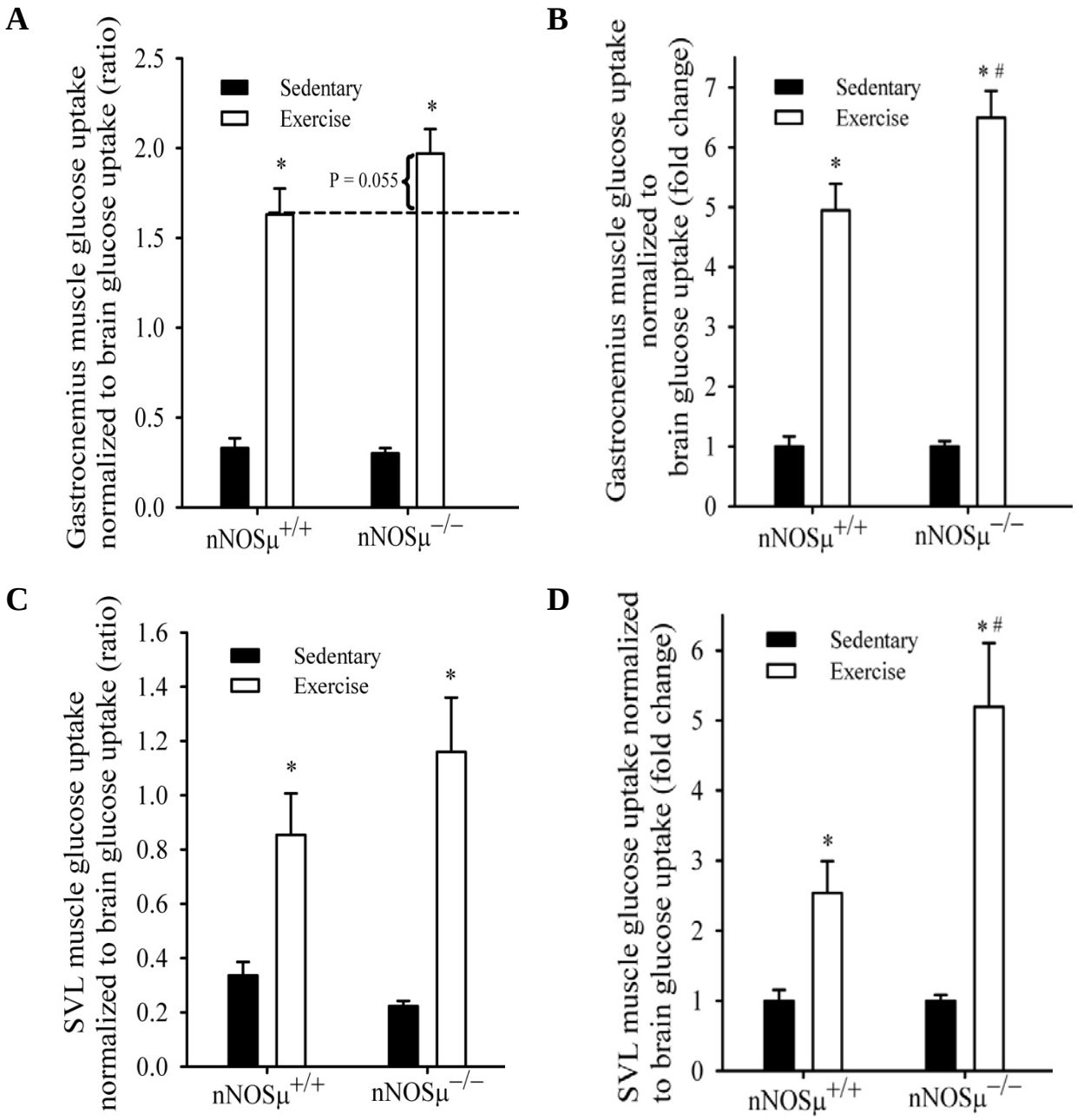
574 Figure 3: Representative blots for TBC1D1, TBC1D1 Ser^{660} phosphorylation and actin (A),
575 gastrocnemius muscle TBC1D1 abundance in sedentary muscles (B), gastrocnemius muscle
576 TBC1D1 Ser^{660} phosphorylation relative to TBC1D1 abundance (C). Data are means ± SEM, n =
577 9 & 4 for sedentary nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ respectively, and 9 & 5 for exercise nNOSμ$^{+/+}$ and
578 nNOSμ$^{-/-}$ respectively. † $P < 0.05$ main effect for exercise.

579

Figure 4: Gastrocnemius muscles eNOS (A) and GLUT4 (B) protein expressions in sedentary state relative to actin and tubulin abundance respectively. Data are means ± SEM; n = 9 for $nNOS\mu^{+/+}$ and 4 for $nNOS\mu^{-/-}$. For GLUT4 protein expression, bands at 45 and 40 kDa represented glycosylated and de-glycosylated GLUT4 respectively. Both bands were used for data analysis.

Figure 5: Gastrocnemius muscle NOS activity at rest (sedentary) and during exercise. Data are means ± SEM; n = 7 & 3 for sedentary $nNOS\mu^{+/+}$ and $nNOS\mu^{-/-}$ respectively; and 7 & 5 for exercise $nNOS\mu^{+/+}$ and $nNOS\mu^{-/-}$ respectively. * $P < 0.05$ vs sedentary of the same genotype; ‡ $P < 0.05$ vs $nNOS\mu^{+/+}$ of the same condition.

591 Figure 1



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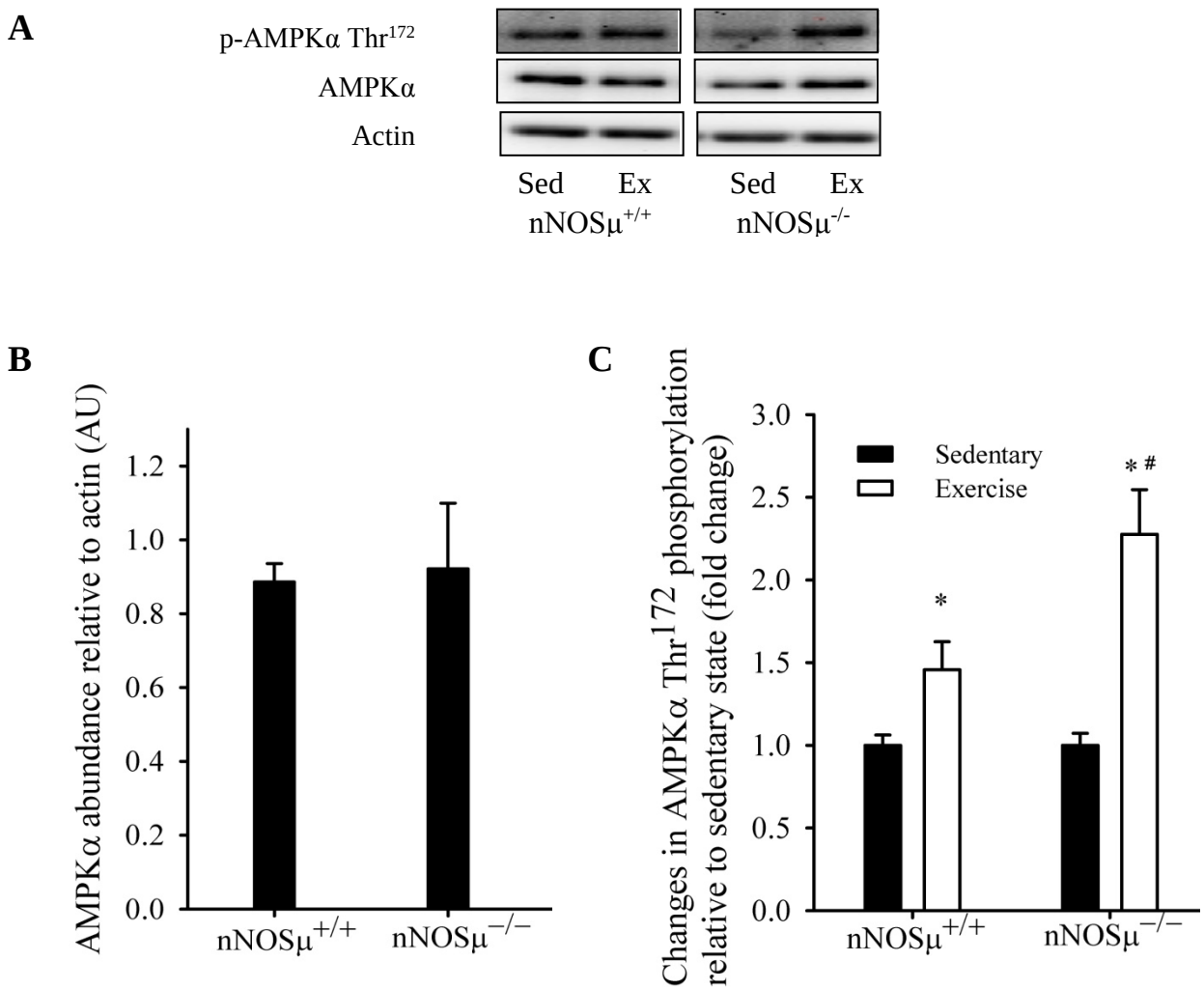
593 Figure 2



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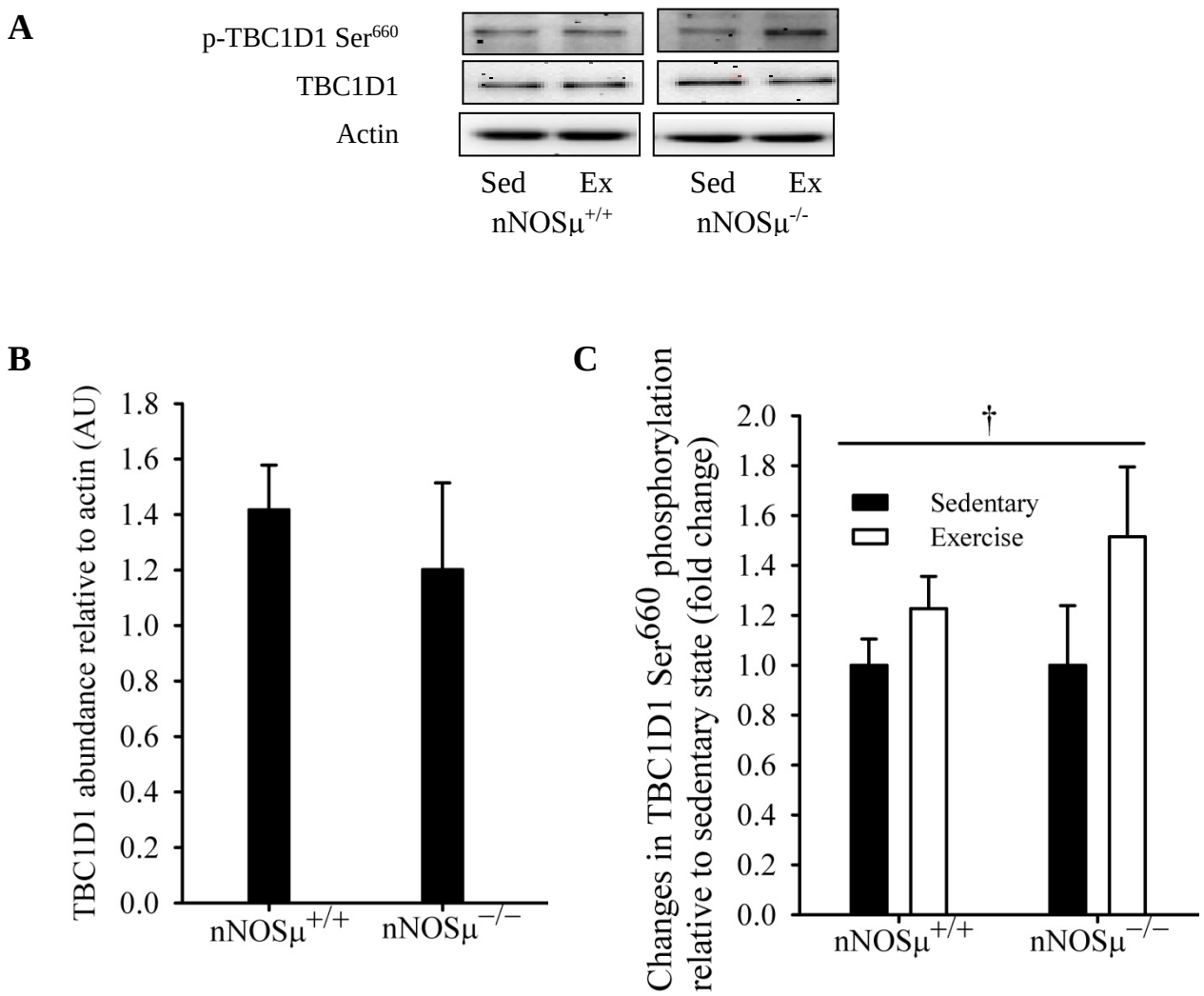
595 Figure 3



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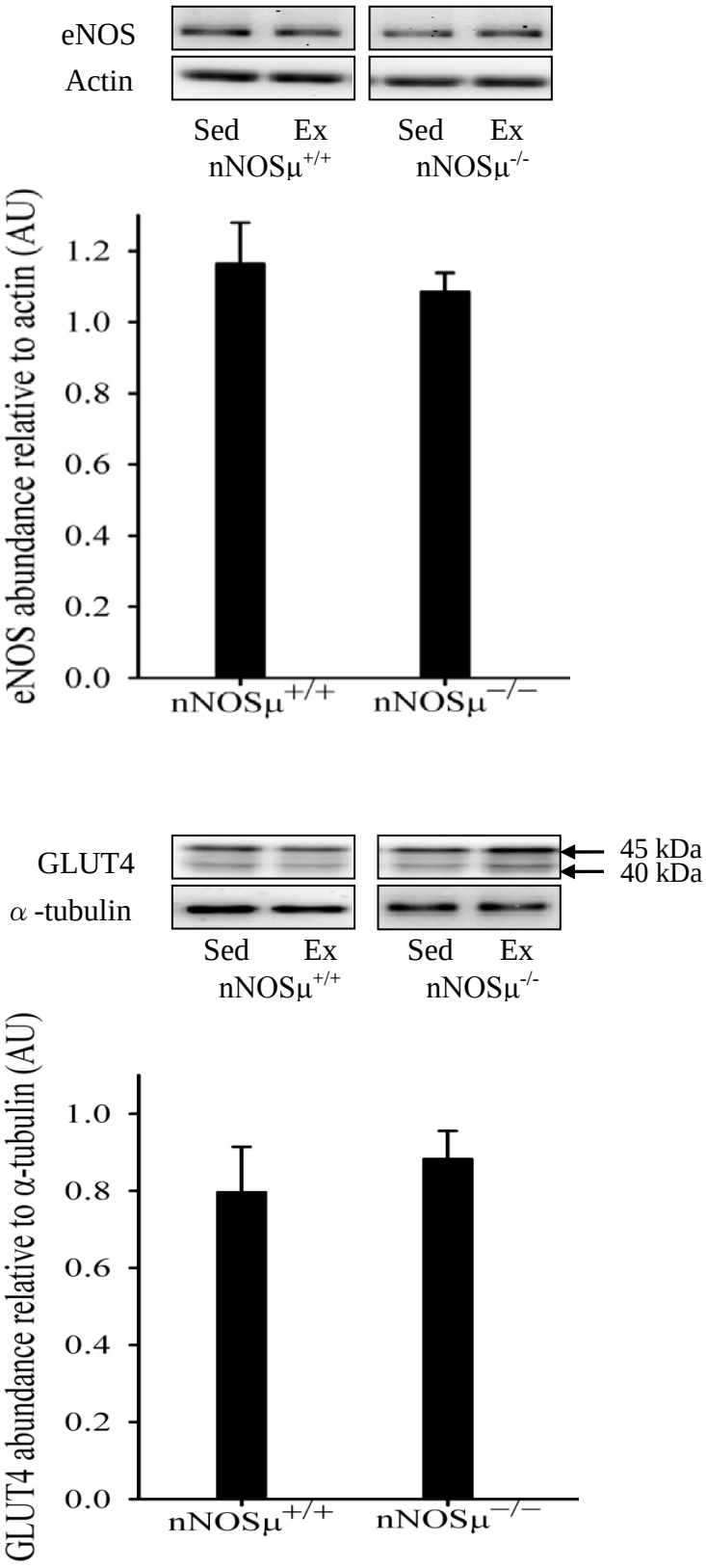
597 Figure 4


A
eNOS
Actin
Sed
Ex
Sed
Ex
nNOSμ+/+
nNOSμ-/-
eNOS abundance relative to actin (AU)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
nNOSμ+/+
nNOSμ−/−
B
GLUT4
α -tubulin
45 kDa
40 kDa
Sed
Ex
Sed
Ex
nNOSμ+/+
nNOSμ-/-
GLUT4 abundance relative to α-tubulin (AU)
1.0
0.8
0.6
0.4
0.2
0.0
nNOSμ+/+
nNOSμ−/−

598 Figure 5

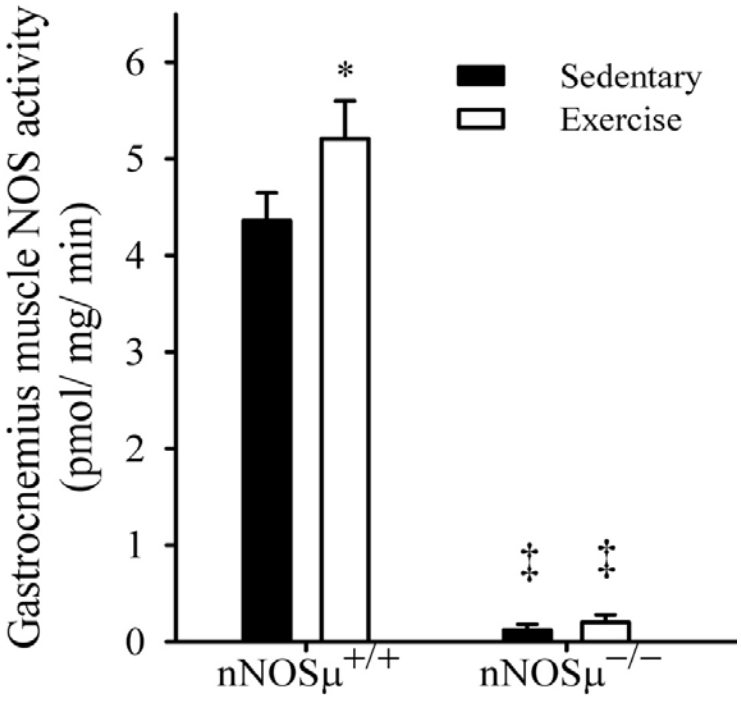


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Table 1: Body weight and exercise capacity of nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ mice

	nNOSμ$^{+/+}$	nNOSμ$^{-/-}$
Male : female (number)	15 : 15	6 : 8
Body weight (g)	29.0 ± 0.8	23.6 ± 1.0 ‡
Max running speed (m/min)	31.5 ± 0.9	29.4 ± 1.2
Max running time (min)	17.7 ± 0.6	15.9 ± 0.9

Values are means ± SEM, n = 30 and 14 for nNOSμ$^{+/+}$ and nNOSμ$^{-/-}$ respectively. ‡ $P < 0.05$ vs nNOSμ$^{+/+}$.